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Application of:	Barbara Ensoli	Confirmation No.:	9400
Application No.:	09/555,534	Art Unit:	1648
Filed:	May 31, 2000	Examiner:	Humphrey, Louise Wang Zhiying
For:	HIV TAT, OR DERIVATIVES THEREOF FOR PROPHYLACTIC AND THERAPEUTIC VACCINATION	Attorney Docket No.:	11340-003-999

SUPPLEMENTAL DECLARATION OF MAURO MAGNANI, Ph.D. UNDER 37 C.F.R. § 1.132

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, MAURO MAGNANI, Ph.D., do declare as follows:

1. I am currently Professor of Biochemistry, Director of Centre of Biotechnology, Vice-Rector of the University of Urbino, Urbino, Italy. I have over thirty years of experience as a biochemist in the research and development of products and applications useful in the biotechnology and pharmaceutical industries. I am included in the official list of professional biologists in Italy with n. 017484 "Ordine Nazionale Biologi," and I am a Technical Director nominated by the "Agenzia Italiana del Farmaco, AIFA" with n. AIDT-19/2005. My education and experience are summarized on my Curriculum Vitae, which is attached hereto as Exhibit 1.

2. I have collaborated with Dr. Barbara Ensoli, who is the inventor of the above-identified application No. 09/555,534 (hereinafter "the '534 application"), in the development of methods to produce recombinant, biologically active Tat protein. I also supervise and have supervised the production of such recombinant, biologically active Tat protein, according to good manufacturing practices (GMP), for use in human clinical trials.

3. This Supplemental Declaration is intended to supplement the Declaration of Mauro Magnani, Ph.D. Under 37 C.F.R. § 1.132 ("First Magnani Declaration") filed May 1, 2007 for the '534 application. In particular, this Supplemental Declaration provides further evidence that one skilled in the art as of December 1, 1997 could obtain a composition containing a biologically active Tat that is pharmaceutically acceptable for administration to a human, based on the teaching of the specification of the '534 application and knowledge common in the art as of December 1, 1997, and using only routine experimentation.

4. I stated in Paragraph 6 of the First Magnani Declaration that it was commonly known in the art as of December 1, 1997 that a combination of purification steps should yield improved purification over a single one of the purification steps, and when isolating a recombinant protein from bacterial cells, improved purification would, for example, decrease levels of endotoxin in the resulting protein preparation, a result known to be desirable when purifying a protein for human therapeutic use. The article by Takacs *et al.*, "Purification of clinical grade proteins produced by recombinant DNA technologies," J Immunol Methods. 1991 Oct 25;143(2):231-40 ("Takacs *et al.*"), a copy of which is attached hereto as Exhibit 2, documents this desirability. The authors of Takacs *et al.* indicate that a combination of purification steps was found to be necessary to reduce endotoxin levels for the production of a clinical grade vaccine candidate (see Takacs *et al.*, paragraph bridging pages 236 and 237) to avoid the "recurrent problem [of endotoxin contamination] in the preparation of solutions for parenteral use in humans" (see Takacs *et al.*, page 237, col. 2, lines 1-3).

5. I stated in Paragraphs 9 and 10 of the First Magnani Declaration that PMSF is a serine protease inhibitor that protects proteins from degradation by serine proteases which are released from the cell along with the protein during purification, and that a person skilled in the art would know that methods to reduce protease activity, such as expressing the protein of interest in a bacterial system that is deficient in proteases or carrying out the initial step(s) of purification at 4°C, which are commonly known in the art, could be employed in order to avoid the use of PMSF. The article by Voet *et al.*, Biochemistry, 2nd Edition, 1995, John Wiley & Sons, Inc. ("Voet *et al.*") at page 73, attached hereto as Exhibit 3, shows that it was known in the art as of December 1, 1997 that purifying a protein at a pH or a temperature that renders proteases inactive but that is not harmful to the protein of interest, *e.g.*, near 0°C, is desirable to prevent damage due to protease digestion (see Voet *et al.*, page 73, col. 1, ¶¶4

and 6). The authors in Voet *et al.* additionally indicate that degradative enzymes such as proteases are eliminated "as the purification of a protein progresses" (see Voet *et al.*, page 73, col. 1, ¶6, last sentence), thus, showing that the increased purification afforded by a combination of purification steps is desirable also for this reason. One skilled in the art as of December 1, 1997 also would know that immediate application of purification procedures to the protein preparation also would reduce protease digestion of the protein being purified, since the more quickly other proteins such as proteases are separated from the protein of interest, the less time there is for proteolytic digestion of the protein to occur.

6. A person skilled in the art as of December 1, 1997 could also avoid the use of PMSF by directly collecting broken cell suspensions into a beaker containing solid guanidine hydrochloride, which, among other things, acts as a protease inhibitor. See page 235, column 2, lines 1-13 of Takacs *et al.* Thus, a broken cell suspension of *E. coli* that recombinantly produces Tat can be solubilized in solid guanidine hydrochloride for purification of biologically active Tat. While Takacs *et al.* describes the solubilization of lysates in solid guanidine hydrochloride calculated for 300 ml of 6 M solution (see page 232, col. 2, ¶2, lines 13-16), in order to carry out subsequent steps of Tat purification, a person skilled in the art as of December 1, 1997 would know that the 6M guanidine hydrochloride solution could be diluted to permit Tat to bind to the chromatographic supports used in subsequent purification steps.

7. It is my judgment and opinion that, as shown by Takacs *et al.* and Voet *et al.*, a person skilled in the art as of December 1, 1997 would know that a combination of purification steps should result in increased purification and thus reduced endotoxin levels and reduced protease activity, and would believe that such combination is desirable. It is also my judgment and opinion that a person skilled in the art as of December 1, 1997 could avoid the use of undesirable chemicals such as PMSF by routine experimentation, and thus, would choose to include heparin affinity chromatography rather than HPLC when choosing a combination of purification steps selected from those taught in the specification of the '534 application.

8. Indeed, to produce the biologically active Tat used in the human clinical trials described in Paragraph 2 above, and described in the Second Declaration of Barbara Ensoli, M.D., Ph.D. Under 37 C.F.R. § 1.132 filed on May 1, 2007 for the '534 application, Tat was

expressed in a protease deficient *E. coli* strain, and purification was achieved by performing ion exchange chromatography and heparin affinity chromatography immediately after cell disruption, with all purification steps being performed at about 4°C.

9. Therefore, in view of the foregoing and further in view of my statements made in the First Magnani Declaration, in my judgment and opinion, a person skilled in the art as of December 1, 1997, based on the teaching of the specification and knowledge common in the art as of December 1, 1997, and using only routine experimentation, could combine the ion-exchange chromatography and heparin affinity chromatography steps as described in the specification in the appropriate order and in the absence of PMSF to obtain a Tat composition that is pharmaceutically acceptable for administration to a human.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application, and any patent issuing thereon.

Date: Oct. 13, 2007

Mauro Magnani

Mauro Magnani, Ph.D.

Curriculum Vitae – Prof. Mauro Magnani

MAGNANI Prof. Mauro, Ph.D. Italian, Professor of Biochemistry
BORN: April 9, 1953, Italy
LANGUAGES: Italian, English
EDUCATION: Univ. Urbino, Italy, Ph.D., 1976
PRIMARY POSITION: Professor of Biochemistry and Director Centre of Biotechnology.
PROFESSIONAL CAREER: Visiting Researcher, Dept. Biochemistry, Univ. Birmingham, 1980; Visiting Prof. Dept. Biolgy, Haifa, Israel, 1983; Asst. Prof. Univ. Urbino, 1977-82, Assoc. Prof., 1982-1986, Prof. 1986 - ; Dean, Faculty of Sciences University of Urbino 1995-2001; Director Interuniversity Consortium for Biotechnology (CIB) 1998-2004; Vice Rector of the University of Urbino 2001- .Include in the official list of professional biologist in Italy with n. 017484 "Ordine Nazionale Biologi". Technical Director nominated by the "Agenzia Italiana del Farmaco, AIFA" with n. AIDT-19/2005.
CURRENT RESEARCH: Development of new drug delivery and drug targeting systems; Protein turnover ubiquitination and regulation of gene expression; Mechanisms of drug resistance and drug toxicity; Modulation of NF-kB and gene expression by oligonucleotide decoys, vaccine development; nanobiotechnology in drug delivery.
PUBLICATIONS: over 300 articles published in international refereed scientific journals; Co-editor of three books:
"*Red Blood Cell Aging*", Plenum Press, N.Y., 1991, pp. 383.
"*The Use of Resealed Erythrocytes as Carriers and Bioreactors*", Plenum Press, N.Y., 1992, pp. 361.
"*Erythrocyte Engineering for Drug Delivery and Targeting*", Landes Bioscience, 2002.
REFEREE: Programmes of the E.U.; The International Science Foundation (U.S.A.); Target Project "Biotechnology" of the National Research Council (C.N.R.).; Member of the Project "Patologia clinica e terapia dell'infezione da HIV" of the Italian Ministry of Health; PRIN and FIRB Projects of Italian Ministry of University and Research; Member of Committee Post Genoma (C.N.R); Include in the "Albo degli Esperti" of M.I.U.R. and Eureka Projects of EU.
REVIEWER: Biotechnology and Applied Biochemistry; Nature Biotechnology; Drugs; Leukemia; European Journal Haematology; Biochimica et Biophysica Acta; Blood; Journal of Cellular Engineering; Journal of Internal Medicine; Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology; Mechanisms of Ageing and Development; Antiviral Research; Journal of Chromatography; Journal of Biological Regulators and Homeostatic Agents; Life Sciences; Biochemistry; International Journal of Biochemistry and Cell Biology; Human Gene Therapy; European Journal of Biochemistry; Clinical Pharmacokinetics; Autoimmunity; Oncogene; Haematologica; J. Controlled Release; Editorial Board: Current Drug Targets, Biotechnology.

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US Patent 5,753,221

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Assignee: Dideco S.p.A., Mirandola, Italy

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Brevetto C.N.R.
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U.Benatti, G. Brandi, E. Garaci, M. Magnani, E. Millo, A.T. Palamara, L. Rossi. "*Derivati del glutathione e loro utilizzo per il trattamento di malattie virali*".

Journal of Immunological Methods, 143 (1991) 231–240

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Preparation of clinical grade proteins produced by recombinant DNA technologies

Bela J. Takacs and Marie-Françoise Girard

Pharma Research Technology, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

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Methods were developed for the production of clinical grade malaria vaccine candidates expressed in *E. coli* by recombinant DNA technologies. The essential features of the purification protocol consist of (1) mechanical breakage of host cells and solubilization of the recombinant proteins in 6 M guanidine hydrochloride; (2) ammonium sulfate fractionation; (3) affinity chromatography on a Ni^{2+}-chelate gel in the presence of 6 M guanidine hydrochloride; and (4) ion exchange chromatography on a Phospho Ultrogel column in the presence of 6 M urea. The use of undesirable chemicals (PMSF, DFP, TFA, acetonitrile, etc.) was avoided rather than demonstrating their complete removal after the purification steps. Testing of chromatographic fractions for host-cell proteins and the elimination of fractions with *E. coli* protein content was found necessary to obtain a final product that contained less than 0.01% of host derived proteins.

The recombinant proteins were renatured either from 8 M urea or from 6 M guanidine hydrochloride by increasing the pH to 10.5 in the presence of glycine and EDTA, reduction with DTT, dilution to a protein concentration below 1 $mg \cdot ml^{-1}$, and dialysis against 0.9% NaCl. The method presented here can be tailor-fit, with minor modification, for the purification of almost any recombinant protein and the final product satisfies current regulations concerning the production of clinically acceptable therapeutic products.

Key words: Recombinant protein; Ni^{2+}-chelate affinity chromatography; Malaria vaccine; Dot-blot analysis; Renaturation

Introduction

Rapid advances in recombinant DNA technology over the past few years have allowed us to clone almost any gene and to express its product in cells growing in culture in large amounts and at reasonable cost. The impressive list of protein products derived from cloned genes include vaccines, enzymes, hormones, immunomodulators and viral antigens. Some of these proteins, for example malaria vaccine candidates for immunization against the sporozoite stage of the parasite, could not have been produced in reasonable amounts by conventional methodologies. Sporozoites can only be obtained in small numbers from the salivary glands of infected mosquitoes, an approach which is not practical for large scale

Correspondence to: B. Takacs, Pharma Research Technology, 69/245, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetraacetic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; EU, endotoxin unit.

vaccine production. The preparation of large amounts of recombinant proteins in highly purified and well-characterized form, therefore, has become a major task for protein chemists. High purity is essential since these proteins are produced in heterologous host cells (bacteria, yeast, insect cells) that are rich in antigens and toxins.

In this communication we present a general protocol for obtaining clinical grade recombinant malaria proteins from *E. coli* either on a laboratory or on an industrial scale. The method is also applicable to other proteins or host cells, and is designed to yield a product which satisfies current regulations concerning the production of clinically acceptable therapeutic products.

Materials and methods

Expression of recombinant proteins

To facilitate protein purification by metal chelate affinity chromatography all of the recombinant malaria proteins were engineered to contain a hexa-His affinity tail either at the amino or at the carboxy terminus. Synthetic oligonucleotides coding for histidines were ligated to the genes coding for the malaria proteins and inserted into pDS6/RBSII plasmids (Stueber et al., 1990). Following transformation into *E. coli* strain M15, W3110 R^-, or SG 13009, transformants were selected on LB agar containing 25 $\mu g \cdot ml^{-1}$ kanamycin and 100 $\mu g \cdot ml^{-1}$ ampicillin. For induction of protein synthesis, cultures (1 to 100 liters) were grown in antibiotic-supplemented L broth to a cell density of $A_{600} = 0.8$, and induced for 3–5 h with 200 $\mu g \cdot ml^{-1}$ of isopropyl-β-D-thiogalactopyranoside.

Metal-chelate affinity chromatography

The metal-chelate affinity adsorbent, nitrilotriacetic acid-Sepharose CL-6B (NTA-Sepharose), was prepared by published procedures (Hochuli et al., 1987). A similar gel, Ni^{2+}-NTA-agarose, together with expression plasmids, is now commercially available from Diagen (cat. no. 30250, Institute for Molecular Biological Diagnostics, P.O. Box 130247, Düsseldorf 13, F.R.G.). Columns were packed with the NTA-Sepharose gel to a bed volume of 60 ml in distilled H_2O and derivatized with Ni^{2+} by applying three column volumes of 0.1 M nickel(II)acetate in 0.1 M ammonium acetate buffer, pH 7.0. Free Ni^{2+} ions were removed by washing the column with 0.5 M NaCl in 0.1 M ammonium acetate buffer, pH 4.0. The column was then equilibrated with Ni^{2+}-chelate column loading buffer (NLB: 6 M guanidine HCl (Ultra Pure, Schwarz/Mann Biotech, Cleveland, OH, U.S.A.); 0.1 M ammonium acetate, pH 7.0; 10% glycerol (Ultra Pure, Bethesda Research Labs., Gaitherburg, MD, U.S.A.)). Sample in NLB was applied and the column was washed with the same buffer until OD_{280} reached a background level. The column was developed by lowering the pH of NLB stepwise to 6.0, 5.5, and 5.0 with acetic acid. The column could be re-used for purification of the same recombinant protein after it had been washed with pH 4.0 NLB and re-equilibrated in pH 7.0 NLB. Either after several runs, or especially if the column was to be used for the purification of a different construct the column was completely regenerated with three column volumes of 50 mM EDTA, pH 8.0, containing 6 M guanidine HCl, followed by three column volumes of 0.5 M NaOH. The column was then washed with d H_2O and derivatized with nickel(II)acetate as described above.

Recombinant protein purification

Each 100 g of *E. coli* paste, expressing a recombinant protein, was washed once with breaking buffer (BB: 50 mM Hepes, pH 8; 20% glycerol; 0.15 M NaCl; 1 mM $MgSO_4$; 2 mM DTT; 5 mM EGTA), and resuspended to 150 ml in BB. The protease inhibitors, Trasylol (intravenous injection grade, from Bayer, Leverkusen, F.R.G.) and ϵ-aminocaproic acid, were added to 100 $U \cdot ml^{-1}$ and 5 mM final concentration, respectively. DNase, 10 $\mu g \cdot ml^{-1}$, was added and the *E. coli* cells were broken in a pre-cooled French pressure cell (SLM Instruments, Urbana, IL, U.S.A.) at 20,000 $lb \cdot in^{-2}$. The lysed cell suspension was collected directly into a beaker containing solid guanidine HCl (calculated for 300 ml of 6 M solution). EDTA was added to 5 mM and the suspension was stirred magnetically for 30 min at 4 °C. The suspension was centrifuged at 23,000 × *g* for 60 min to obtain a crude guanidinium HCl extract. The pellet was

discarded and 4 M (here defined as saturated) ammonium sulfate solution was added to the supernatant fraction. The amount of ammonium sulfate, predetermined by pilot experiments, was that amount which precipitated only a small per cent of the recombinant protein in question. Depending on the construct, this amount was found to represent 30–50% of ammonium sulfate saturation. The precipitated material, sometimes representing as much as 60% of the total *E. coli* proteins, was pelleted by centrifugation at 16,000 $\times g$ for 15 min. The supernatant fraction, containing essentially all of the recombinant protein, was dialysed against 80% saturated (3.2 M) ammonium sulfate for 16 h at 4 °C. The precipitate was pelleted by centrifugation at 10,000 $\times g$ for 15 min, and the pellet was dissolved in and dialysed against NLB to remove ammonium sulfate. The dialysed solution was centrifuged at 36,000 $\times$ g for 30 min and filtered through a 0.8 μm pore size membrane prior to application on to a 60 ml NTA-Sepharose column, derivatized with nickel (II)acetate and equilibrated in NLB. The column was washed with NLB at 0.8 ml $\cdot$ min^{-1} until OD_{280} reached a background level. Washing was continued by lowering the pH of the NLB stepwise to 6.0, 5.5, and 5.0.

Fractions from the Ni^{2+}-chelate column that contained recombinant protein were pooled and dialysed against 80% saturated ammonium sulfate for 16 h at 4 °C. Precipitated proteins were pelleted by centrifugation at 16,000 $\times g$ for 15 min. Pellets were dissolved in buffer A (6 M urea; 20 mM sodium acetate, pH 5.0; 2 mM EDTA; 10% glycerol) and dialysed against buffer A for 16 h at 4 °C. The dialysed solution was centrifuged at 36,000 $\times g$ for 15 min and the supernatant fraction was applied on to a 30 ml Phospho-Ultrogel (IBF, Villeneuve-la-Garenne, France) column, equilibrated in buffer A. The column was washed with buffer A until OD_{280} reached a background level, and developed with a 10 h linear gradient of buffer B (buffer A + 0.5 M NaCl) at 0.5 ml $\cdot$ min^{-1}. Fractions containing the 'purest' form of the recombinant proteins, determined by SDS-PAGE and dot-blot analysis for *E. coli* proteins, were pooled and concentrated by dialysis against 80% saturated ammonium sulfate.

Analysis of the Ni^{2+}-chelate column fractions

Guanidine HCl forms an insoluble precipitate with SDS. Therefore prior to SDS-PAGE analysis of the fractions that eluted from the Ni^{2+}-chelate column, guanidine HCl had to be removed. An aliquot (100 μl) from each fraction to be analysed was transferred to a conical glass tube and diluted to 1.0 ml with water. An equal volume of 30% (w/v) TCA was added and the tubes were vortexed. Precipitates were pelleted by centrifugation at 2000 $\times g$ for 5 min. Supernatants were aspirated and the pellets were washed with 5 ml of cold acetone to remove TCA. Precipitated proteins were dispersed in 100 μl of 25 mM Tris-HCl, pH 7.5; 1% Triton X-100, prior to the addition of one half volume of a three times concentrated SDS-sample buffer solution (Takacs, 1978). Tubes were incubated in a boiling water bath for 1 min and 10–20 μl were applied from each fraction to the sample compartment of 0.8 mm thick SDS slab gels (Takacs, 1979).

Verification of identity of the recombinant proteins

Identity of the recombinant proteins was verified by immunoblotting experiments (Takacs and Staehli, 1987), as well as by their ability to induce antibodies against parasites. Mouse monoclonal antibodies, known to react with the recombinant proteins in question, were a generous gift of Dr. H. Matile.

Dot-blot analysis of chromatographic fractions for E. coli proteins

Antisera against *E. coli* proteins were prepared by immunizing rabbits with protein derived from the same *E. coli* strain that was used for the recombinant protein expression. Bacteria harboring the same plasmid, but lacking the gene for the recombinant protein, were subjected to the initial steps of the same purification protocol that was used for the purification of the recombinant protein. *E. coli* proteins that were eluted from the Ni^{2+}-chelate affinity column at the pH at which the major part of the recombinant protein eluted (in this case pH 5.5), were used to immunize rabbits.

10 μl of each chromatographic fraction to be analysed were spotted on to a sheet of reinforced, 0.2 μm pore size, nitrocellulose membrane (from

Sartorius, Göttingen, F.R.G.), cut to a size of 10 × 16 cm. The membrane was pre-soaked in distilled H_2O and blotted between two sheets of Whatman no. 3 chromatographic paper prior to application of the samples. After a few minutes of air-drying, unoccupied sites on the nitrocellulose were blocked by incubating the membrane in 100 ml of 'blocking buffer' (Takacs and Staehli, 1987) (3% HiPure liquid gelatin (Norland Products, U.S.A.); 20 mM sodium borate, pH 8.5; 0.3 M NaCl; 0.05% (v/v) Tween 20; 1% (w/v) casein hydrolysate; 5 mM EDTA; 0.02% (w/v) NaN_3) at room temperature for 60 min on a rocking platform. The blocking buffer was then replaced by rabbit anti-'normal' *E. coli* antiserum, diluted 1/250 in blocking buffer, and incubation continued for 16 h at room temperature. The nitrocellulose membrane was washed for 4 × 15 min in 'wash buffer' (Takacs and Staehli, 1987) (50 mM sodium borate, pH 8.5; 0.3 M NaCl; 0.05% Tween 20; 0.1% HiPure liquid gelatin; 0.1% casein hydrolysate; 5 mM EDTA; 0.05% merthiolate), and incubated in ^{125}I-labelled protein G (Amersham, Buckinghamshire, England) for 60 min at room temperature. The ^{125}I-protein G was diluted in blocking buffer supplemented with 5 mM KI, to contain approximately 3–4 × 10^5 cpm · ml^{-1}. The membrane was washed again 4 × 15 min in wash buffer before it was dried and exposed at −70 °C to RX Medical X ray film (Fuji, Japan) with Cronex intensifying screen (Du Pont, Wilmington, DE, U.S.A.).

Renaturation of recombinant proteins

Recombinant protein solutions either in 6 M guanidine HCl or in 6–8 M urea were diluted to a protein concentration between 0.5–1.0 mg · ml^{-1} with 50 mM glycine-OH, pH 9.0; 5 mM EDTA. The pH of the solution was adjusted to 10.5 with NaOH. DTT was added to 5 mM and the solution was dialysed against 50 mM glycine-OH, pH 10.5; 5 mM EDTA, at room temperature for 3 h. Dialysis was then continued against several changes of saline (0.9% NaCl) at 4 °C. An aliquot of the dialysed solution was filter sterilized through a 0.22 μm pore-size membrane (Millex-GV from Millipore, Molsheim, France) and protein concentrations before and after filtration were determined.

Quantitation of E. coli protein contamination

E. coli protein content of the recombinant malaria vaccine candidates was estimated by dot-blotting experiments. Three-fold serial dilutions were made of known amounts of both 'normal' *E. coli* proteins and of the recombinant protein preparations in 3 M urea; 0.1% BSA; 5 mM EDTA; 50 mM glycine-OH, pH 9.0. 5 μl, representing known amounts of protein, from each dilution were spotted on to a sheet of nitrocellulose membrane. The membrane was then treated as described above for the dot-blotting analysis of chromatographic fractions. The level of *E. coli* protein content in the recombinant protein preparations was estimated by comparing the intensities of the autoradiographic spots.

Endotoxin content was determined by the *Limulus amebocyte* lysate (LAL) test (Friberger, 1982).

Adsorption and desorption of vaccine candidates to or from Al(OH)$_3$ adjuvant

To 100–400 μg of sterile recombinant protein solution in 877 μl of saline, 123 μl of Alhydrogel (Superfos Biosector, Vedbaek, Denmark) was added aseptically. The gel was mixed and left at 4 °C overnight. The affinity of most recombinant proteins to $Al(OH)_3$ adjuvant was found to be very strong since they could not be desorbed by incubating $Al(OH)_3$ pellets in SDS-sample buffer for 5 min at 100 °C. It was found, however, that essentially complete protein recovery was possible by incubating $Al(OH)_3$ pellets in buffers containing high concentration of phosphate (0.9 M) or tri-sodium citrate (0.4 M). To test for stability of the vaccine candidates adsorbed to $Al(OH)_3$ and incubated for various times at different temperatures, the $Al(OH)_3$ gel was pelleted by centrifugation at 8000 × *g* for 5 min. The supernatant fraction was assayed for protein using Coomassie protein assay reagent (Pierce, Rockford, IL, U.S.A.), and by SDS-PAGE. The pellet was resuspended in 100 μl of desorbing buffer (0.4 M tri-sodium citrate; 2% lithium dodecyl sulfate; 10% glycerol; 5% 2-mercaptoethanol; 0.003% bromphenol blue, pH adjusted to 6.8 with citric acid) and incubated for 3 min in a boiling water bath. 10–25 μl of the mixture was trans-

ferred directly into each well of 0.8 mm thick SDS slab gels.

Results and discussion

Purification of recombinant proteins

Recombinant DNA technology was used to produce malaria vaccine candidates with either N terminal or C terminal polyhistidine affinity tails. For the purpose of demonstrating the validity of the purification protocol we present the preparation of two clinical grade malaria vaccines, CSP-His_6 and His_6-MECS. CSP-His_6 represents sequences from the circumsporozoite protein present on the surface of the sporozoite stage of the malaria parasite, *Plasmodium falciparum*. His_6-MECS is a recombinant fusion protein that contains elements from both the sporozoite and blood stages (merozoites) of the parasite.

For laboratory scale purification of recombinant proteins, 100–200 g of *E. coli* paste was found to represent a convenient amount of starting material. Mechanical breakage was used to open the cells in the presence of DNase and the protease inhibitors Trasylol and ϵ-aminocaproic acid. The use of protease inhibitors that are not approved by the regulatory authorities (see Esber, 1985), such as PMSF or DFP was avoided. The widely used PMSF could modify amino acid side chains on proteins, which could lead to reduced efficacy of the antigenic material itself and might elicit cross-reactive autoimmune responses.

The broken cell suspension was collected directly into a beaker containing solid guanidine HCl. The use of this strong chaotrope has several advantages; it efficiently solubilizes recombinant proteins present in inclusion bodies; it acts as a protease inhibitor; it can easily be removed by dialysis; and it is compatible with the subsequent Ni^{2+}-chelate affinity chromatography step described in this article.

The guanidine HCl extracts were subjected to ammonium sulfate fractionation. It was found that at 30% ammonium sulfate saturation (in the presence of guanidine HCl) the recombinant malaria protein, CSP-His_6, remained soluble. The recombinant malaria fusion protein, His_6-MECS, remained soluble even at 50% ammonium sulfate saturation, whereas over 60% of the proteins present in the original guanidine HCl extract precipitated and were removed by a simple cen-

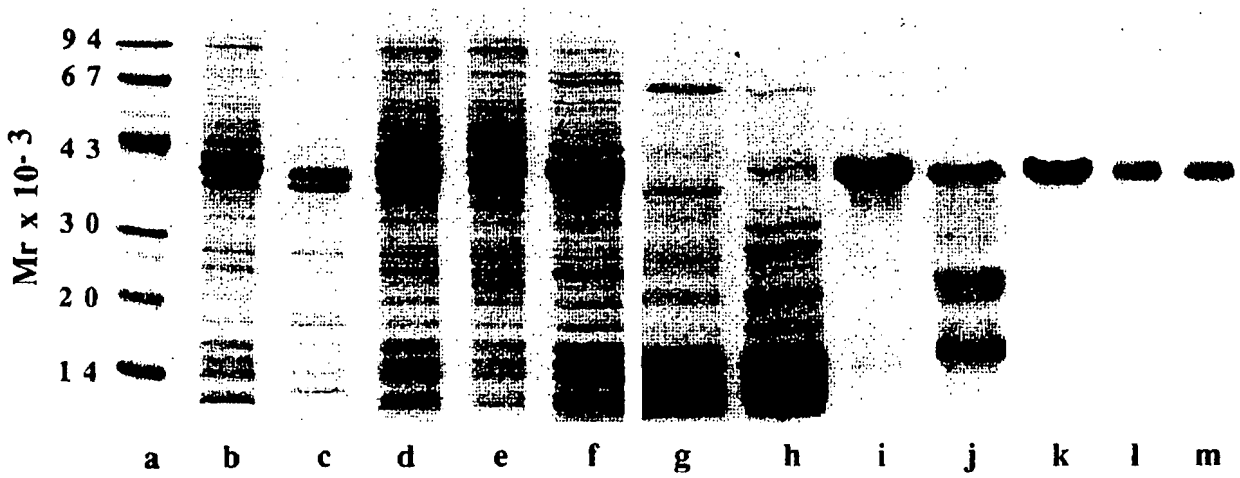

Fig. 1. SDS-PAGE analysis of purification of recombinant malaria fusion protein, His_6-MECS, which contains elements of both the merozoite and sporozoite stage of the parasite. Molecular weight standards are shown in lane *a*. Lane *b* represents total bacterial extract; lanes *c* and *d* contain 6 M guanidine HCl insoluble and soluble fractions, respectively. Lanes *e* and *f* represent 50% saturated ammonium sulfate insoluble and soluble fractions, respectively. Lane *f* also represents the material that was applied on to the Ni^{2+}-chelate column. Material eluting from the Ni^{2+}-chelate column at pH 7 is shown in lane *g*, at pH 6 in lane *h*, at pH 5.5 in lane *i* and at pH 5 in lane *j*. Lane *k* contains His_6-MECS after the Phospho-Ultrogel purification step. Lanes *l* and *m* show immunoblot analysis of His_6-MECS, using antibodies reactive with the sporozoite and merozoite part of the antigen, respectively.

trifugation step. The advantage of this quick separation method is demonstrated in Fig. 1. The recombinant protein, His_6-MECS, is barely visible in the total cell lysate, but is highly enriched in the 50% saturated ammonium sulfate supernatant (Fig. 1*f*). Ammonium sulfate is a well-established protein precipitant and it stabilizes proteins. Montoya and Castell (1987), for example, studying the long-term storage of peroxidase-labelled antibodies, found that when such conjugates were stored as ammonium sulfate precipitates at 4 °C, both enzymatic and immunological activities had an estimated half life of nine years.

The ammonium sulfate-soluble recombinant protein fractions were then subjected to dialysis against 80% saturated ammonium sulfate. Precipitated proteins (Figs. 1*f* and 2*d*) were dissolved in and dialysed against 6 M guanidine HCl buffer for the Ni^{2+}-chelate affinity chromatography step. The quadridentate metal chelate adsorbent used in these purification steps has a remarkable selectivity for proteins or peptides containing neighboring histidine residues (Hochuli et al., 1988). The binding of histidine-containing proteins to this Ni^{2+}-chelate gel is pH-dependent and is stable between pH 8 and pH 6 even in the presence of 6 M guanidine HCl. Most of the contaminating *E. coli* proteins were washed through the column at pH 7 without any appreciable loss of the recombinant proteins (Figs. 1*g* and 2*e*). The recombinant proteins were then eluted from the column at pH 5.5 (Figs. 1*i* and 2*g*). Essentially all the protein remaining on the column was eluted at pH 5.0 since lowering the pH to 4.0 or regenerating the column with EDTA did not result in the elution of proteinaceous material. In the experiments described here we used pH-dependent elution of the recombinant proteins from the Ni^{2+}-chelate columns. Other elution methods, for example with buffers containing histidine or imidazol, might be advantageous for proteins sensitive to low pH.

After the Ni^{2+}-chelate purification step the recombinant proteins are well over 90% pure, as judged by SDS-PAGE analysis (Figs. 1*i* and 2*g*). This purity might be acceptable for many applications, but for the production of clinical grade vaccine candidates additional purification steps

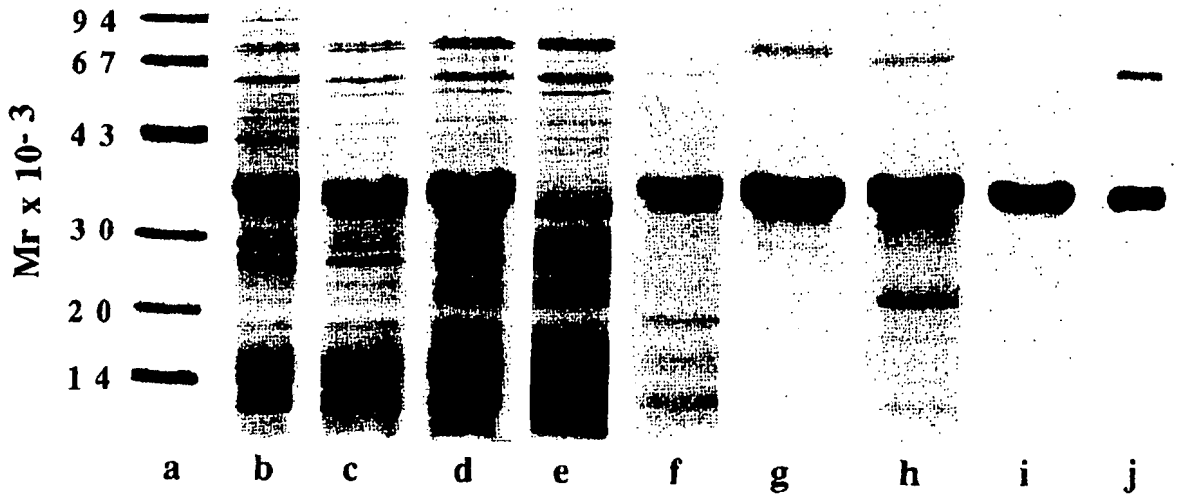

Fig. 2. SDS-PAGE analysis of purification of recombinant malaria protein, $CSP\text{-}His_6$. Molecular weight standards are shown in lane *a*. Lane *b* contains total bacterial extract. Lane *c* represents proteins that precipitated between 30% and 80% saturated ammonium sulfate. Lanes *d*–*h* represent Ni^{2+}-chelate column fractionation of $CSP\text{-}His_6$; lane *d* contains material applied on to the column; lanes *e*–*h*, material eluted from the Ni^{2+}-chelate column at pH 7, pH 6, pH 5.5 and pH 5, respectively. Lane *i* contains $CSP\text{-}His_6$ after the Phospho-Ultrogel purification step. Lane *j* shows immunoblot analysis of $CSP\text{-}His_6$ using mouse anti-sporozoite antibodies.

were found necessary. After the Ni^{2+}-chelate column fractionation, recombinant proteins were further purified by Phospho-Ultrogel column chromatography in the presence of 6 M urea and 10% glycerol. Glycerol was included because it is known to minimize hydrophobic interactions with the resin and to stabilize proteins (Muto and Tan, 1985; Wellig et al., 1987). At pH 5.0, both of the recombinant proteins described here bound to this strong cation exchange resin and could be eluted with a salt gradient (Figs. 1*k* and 2*i*). Most of the endotoxins that remained associated with the recombinant proteins after the Ni^{2+}-chelate chromatography step were found to pass through freely the Phospho Ultrogel column. Conversely, in pilot experiments, we found that endotoxins adsorbed very strongly to Q-Sepharose FF anion exchange resin (Pharmacia, Uppsala, Sweden), and usually required much higher salt concentration for their elution than the recombinant proteins did. Endotoxin contamination is a recurrent problem in the preparation of solutions for parenteral use in humans. Endotoxins can activate monocyte-macrophage cells to produce a number of biologically active factors including interleukin-1 (Durum et al., 1985), interleukin-6 (Tosato et al., 1988), tumor necrosis factor (Beutler et al., 1985), prostaglandins (Taffet et al., 1982), and leukotrienes (Aderem et al., 1986). Some of these factors may have deleterious physiological effects culminating in fever, endotoxic shock and acute-phase reaction. The sensitivity of mammals to endotoxins is extraordinary since as little as 1 ng (10 EU) can elicit strong fever, shock and even death in susceptible individuals (Nelsen, 1978).

An essential feature of the purification protocol described here is the testing of each chromatographic fraction not only for the presence of the recombinant protein in question but also for

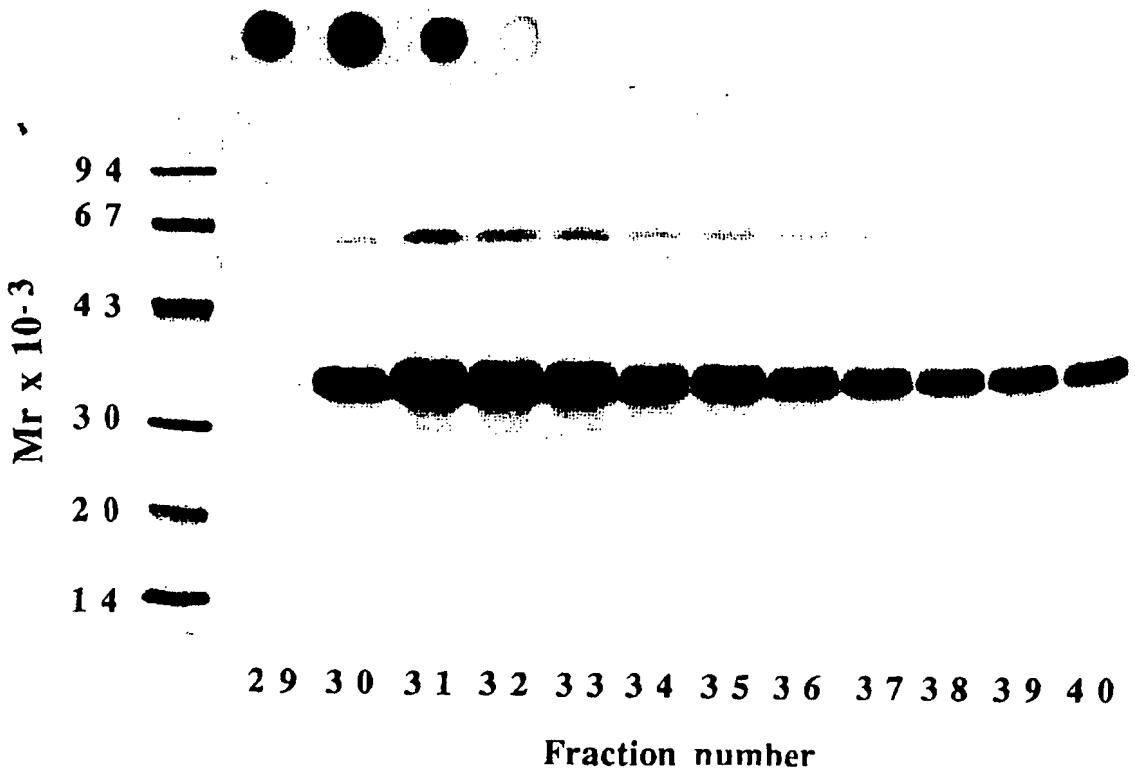

Fig. 3. Coomassie blue-stained SDS-PAGE analysis of CSP-His_6 fractions eluted from the Ni^{2+}-chelate column. Molecular weight standards are shown on the left. Dot-blot analysis for *E. coli* proteins of representative fractions is shown on top.

contaminating *E. coli* proteins. On a sheet of nitrocellulose membrane (10 × 16 cm) as many as 100 chromatographic fractions can easily be tested for the presence of *E. coli* proteins by a simple dot-blot analysis described in the materials and methods section.

Fractions from the Phospho-Ultrogel column that gave a strong positive signal for *E. coli* proteins on dot-blot analysis were not included in the final pool. In this way *E. coli* protein content of the two malaria vaccine candidates described here was reduced to 0.01–0.05%. To reduce the *E. coli* protein content to less than 0.01%, a second Ni^{2+}-chelate column fractionation was performed. It was found that at least 600 mg of the poly-histidine containing recombinant proteins adsorbed quantitatively to the 60 ml Ni^{2+}-chelate column. No detectable OD_{280} absorbing material was eluted with the pH 7.0 NLB. At pH 6.0 a small peak of OD_{280} absorbing material was observed, containing mainly *E. coli* proteins (data not shown). At pH 5.5 essentially all of the recombinant proteins were eluted. SDS-PAGE and dot-blot analysis for *E. coli* proteins are shown in Fig. 3. Based on SDS-PAGE analysis alone, we would have pooled fractions 30–40. However, based on dot-blot analysis for *E. coli* proteins, fractions 30 and 31 were not included in the final pool. This approach has allowed us to reduce the *E. coli* protein content of the final, clinical grade malaria vaccine candidates to an acceptable level of less than 0.01% (Fig. 4). We would like to recommend that such dot-blot assays for *E. coli*, or other host proteins, should be included in all purification schemes aimed at obtaining clinical grade proteins produced by recombinant DNA technology.

Verification of identity of the various constructs was done by immunoblotting experiments (Figs. 1*l*, 1*m* and 2*j*), using antibodies that are known to react with the recombinant proteins. Such immunoblots might also provide us with information as to the presence of aggregated and/or degraded forms of the antigens. However, the pattern and intensity of the reactivities are usually not quantitative, since some antibodies might react preferentially with aggregated forms (dimers) of the antigens (Fig. 2*j*).

We did not make any effort to remove the poly-histidine affinity tails from these malaria vaccine candidates since until now we have not detected the production of autoantibodies, due to these constructs, in animals models. However, if it is desirable to convert such recombinant proteins to their 'natural' counterparts, the introduction of cleavable linker sequences might be undertaken by genetic engineering methods (Sharma, 1986; Hochuli et al., 1988).

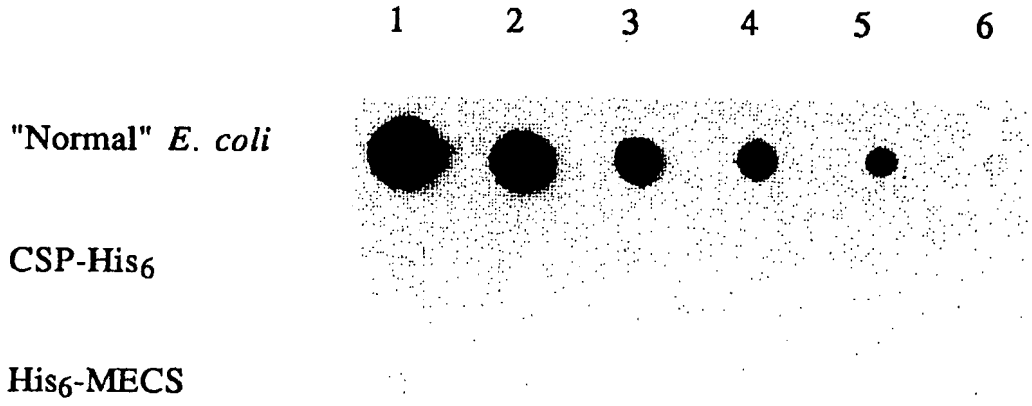

Fig. 4. Quantitation of *E. coli* protein content in CSP-His_6 and His_6-MECS by dot-blotting. Three-fold serial dilutions of 'normal' *E. coli* protein solution was spotted on to nitrocellulose membrane to represent amounts of (1) 120 ng; (2) 40 ng; (3) 13.3 ng; (4) 4.44 ng; (5) 1.48 ng; and (6) 0.49 ng. Amounts of purified recombinant CSP-His_6 and His_6-MECS applied in spot (1) were 6.4 μg and 27.5 μg, respectively.

Renaturation of recombinant proteins

Each of the recombinant proteins whose purification is described here contains four cysteine residues, which could give rise to two disulfide bonds and to a theoretical random pairing of three. At high protein concentrations intermolecular disulfide bond formation is favored (Marston, 1986). Indeed, we have observed that in urea at a protein concentration of 10 $mg \cdot ml^{-1}$ these proteins formed gels, which could be solubilized by the addition of DTT (25 mM). In order to obtain a filter-sterilizable vaccine we had to refold the reduced and denatured recombinant proteins under conditions which favor the formation of intramolecular disulfide bonds. Our studies on refolding recombinant proteins have led to the following three recommendations: (1) during refolding protein concentration must be low, 0.5–1 $mg \cdot ml^{-1}$, in order to favor intramolecular interactions in preference to intermolecular interactions; (2) exposure of the recombinant proteins to alkaline pH (> 9.0) in the presence of thiol reagents (2-mercaptoethanol or dithiothreitol), to solubilize aggregates; and (3) slow removal of the strong denaturant (8 M urea of 6 M guanidine HCl) that was used in the solubilization step by dilution or preferentially by dialysis. The use of dithiothreitol in the reduction step is preferred since it does not form mixed disulfides with proteins, as 2-mercaptoethanol does (Cleland, 1964). We included glycine in the initial steps of renaturation because of its remarkable solubilizing properties. Hansson (1968, 1970) has shown that the aggregation of immunoglobulins (IgG) on freezing can be effectively prevented by glycine. Furthermore, glycine prevented the precipitation of IgG even in the presence of 9.3% polyethylene glycol (PEG), at neutral pH, whereas without glycine IgGs started to precipitate at a PEG concentration of somewhat above 3%. In the presence of glycine even human IgA and IgM remained soluble provided the pH was increased to 9.0 (Hansson et al., 1989).

The renaturation protocol described here is suitable for proteins displaying disulfide-dependent denaturation. Intrinsically insoluble proteins that contain extensive stretches of hydrophobic amino acid residues in their primary sequence might require other treatments to obtain a filter-sterilizeable and injectable product. For proteins that fall into this category, β cyclodextrin or glycocholate (< 1 $mg \cdot ml^{-1}$) might be tried.

By using the four-step purification protocol described in this article we were consistently able to obtain recombinant proteins that contained less than 0.01% of host derived proteins, less than 10 pg of DNA and much less than the upper limit of 300 EU per dose of vaccine (100–400 μg recombinant protein). The integrities of the proteins were always greater than 95% and the vaccine candidates contained less than 5% of degraded or aggregated forms of the antigens.

Acknowledgements

It is a pleasure to thank Dr. J.R.L. Pink for reading the manuscript and Dr. D. Gillessen for providing many valuable comments throughout the course of this work. Drs. D. Stueber and P. Caspers expressed the recombinant malaria proteins in *E. coli* and Dr. Wipf scaled up the production to 100 l fermenter level. Dr. E. Hochuli has kindly provided us with the NTA-Sepharose. We would like to thank them and those mentioned in the text for their contributions.

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DONALD VOET
University of Pennsylvania

JUDITH G. VOET
Swarthmore College

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rating the subcellular assembly from the rest of the cellular material. This is usually accomplished by **differential centrifugation,** a process in which the cell lysate is centrifuged at a speed that removes only the cell components denser than the desired organelle followed by centrifugation at a speed that spins down the component of interest. The required protein is then usually separated from the purified subcellular component by extraction with concentrated salt solutions or, in the case of proteins tightly bound to membranes, with the use of detergent solutions or organic solvents, such as butanol, that solubilize lipids.

C. Stabilization of Proteins

Once a protein has been removed from its natural environment, it becomes exposed to many agents that can irreversibly damage it. These influences must be carefully controlled at all stages of a purification process or the yield of the desired protein may be greatly reduced or even eliminated.

The structural integrity of many proteins is sensitive to pH as a consequence of their numerous acid-base groups. To prevent damage to biological materials due to variations in pH, they are routinely dissolved in buffer solutions effective in the pH range over which the material is stable.

Proteins are easily **denatured** (destroyed) by high temperatures. Although the thermal stabilities of proteins vary widely, many of them slowly denature above 25°C. Therefore, the purification of proteins is normally carried out at temperatures near 0°C. However, there are numerous proteins that require lower temperatures, some even lower than −100°C, for stability. Conversely, some **cold-labile** proteins become unstable below characteristic temperatures.

The thermal stability characteristics of a protein can sometimes be used to advantage in its purification. A heat-stable protein in a crude mixture can be greatly purified by briefly heating the mixture so as to denature and precipitate most of the contaminating proteins without affecting the desired protein.

Cells contain **proteases** (enzymes that cleave the peptide bonds of proteins) and other degradative enzymes that, upon lysis, are liberated into solution along with the protein of interest. Care must be taken that the protein is not damaged by these enzymes. Degradative enzymes may often be rendered inactive at pH's and temperatures that are not harmful to the protein of interest. Alternatively, these enzymes can often be specifically inhibited by chemical agents without affecting the desired protein. Of course, as the purification of a protein progresses, more and more of these degradative enzymes are eliminated.

Some proteins are more resistant than others to proteolytic degradation. The purification of a protein that is particularly resistant to proteases may be effected by maintaining conditions in a crude protein mixture under which the proteolytic enzymes present are active. This so-called **autolysis** technique simplifies the purification of the resistant protein because it is generally far easier to remove selectively the degradation products of contaminating proteins than it is the intact proteins.

Many proteins are denatured by contact with the air-water interface and, at low concentrations, a significant fraction of the protein present may be lost by adsorption to surfaces. Hence, a protein solution should be handled so as to minimize frothing and kept relatively concentrated. There are, of course, other factors to which a protein may be sensitive, including the oxidation of cysteine residues to form disulfide bonds; heavy metal contaminants, which may irreversibly bind to the protein; and the salt concentration and polarity of the solution, which must be kept within the stability range of the protein. Finally, many microorganisms consider proteins to be delicious, so proteins should be stored under conditions that inhibit the growth of microorganisms.

D. Assay of Proteins

To purify any substance, some means must be found for quantitatively detecting its presence. A protein rarely comprises more than a few percent by weight of its tissue of origin and is usually present in much smaller amounts. Yet, much of the material from which it is being extricated closely resembles the protein of interest. Accordingly, an assay must be specific for the protein being purified and highly sensitive to its presence. Furthermore, the assay must be convenient to use because it is done repeatedly at every stage of the purification process.

Among the most straightforward of protein assays are those for enzymes that catalyze reactions with readily detectable products. Perhaps such a product has a characteristic spectroscopic absorption or fluorescence that can be monitored. Alternatively, the enzymatic reaction may consume or generate acid so that the enzyme can be assayed by acid-base titrations. If an enzymatic reaction product is not easily quantitated, its presence may still be revealed by further chemical treatment to yield a more readily observable product. Often, this takes the form of a **coupled enzymatic reaction,** in which the product of the enzyme being assayed is converted, by an added enzyme, to an observable substance.

Proteins that are not enzymes may be assayed through their ability to bind specific substances or the observation of their biological effects. For example, receptor proteins are often assayed by incubating them with a radioactive molecule that they specifically bind, passing the mixture through a protein-retaining filter, and then measuring the amount of radioactivity bound to the filter (Section 34-4B). The presence of a hormone may be revealed by its effect on some standard tissue sample or on a whole organism. The latter type of assays are ususally rather lengthy procedures